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Prognostic Significance of the S-phase Fraction of Light-Chain-Restricted Cytoplasmic Immunoglobulin (cIg) Positive Plasma Cells in Patients with Newly Diagnosed Multiple Myeloma Enrolled on Eastern Cooperative Oncology Group Treatment Trial E9486

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The bone marrow plasma cell labeling index (PCLI) as measured by bromodeoxyuridine uptake is a well-established independent prognostic factor for patients with newly diagnosed multiple myeloma, but the test is not easily done in most laboratories. The purpose of this study was to determine if the proliferative activity (% S-phase) as determined by two-color flow cytometry for cytoplasmic immunoglobulin (cIg) light chain and DNA content also had prognostic significance. As part of Eastern Cooperative Oncology Group clinical trial E9486, 500 patients had successful performance of the bone marrow PCLI. Of 349 patients who had flow cIg and DNA content cytometry, 210 had adequate data to reliably calculate S-phase %. Patients with low % S-phase fraction (<2%) had a significant overall survival advantage over patients high % S-phase fraction (≥2%), median survivals 4.1 vs. 2.9 years (P = 0.032). Measurement of the S-phase % by flow cytometry gives significant prognostic information in patients with newly diagnosed myeloma. However, in multivariate analysis, S-phase % did not add prognostic information when PCLI was in the model. S-phase % added prognostic information only when all cases with flow measurement of S-phase % were included, and when PCLI was excluded from the model. Discriminating a population of only cIg positive cells proved difficult in patients with a low percentage of bone marrow plasma cells. Methodology to measure S-phase % in patients with a low percent plasma cells is needed before this technique can be used for diagnosis and prognosis in myeloma. Am. J. Hematol. 61:232–237, 1999.

Key words: multiple myeloma; s-phase; flow cytometry; labeling index

INTRODUCTION

Proliferative activity of bone marrow plasma cells, as measured by the plasma cell labeling index (PCLI), is a well-established independent prognostic factor for patients with newly diagnosed multiple myeloma [1–6]. The PCLI is also a useful diagnostic tool in differentiating between monoclonal gammopathy of undetermined significance (MGUS), smoldering myeloma and overt multiple myeloma [7,8], and has prognostic value in pri-

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mary systemic amyloidosis [9]. Early PCLI assays utilized [^{3}H]-thymidine incorporation, but technical difficulties and delay in reporting results prevented wide spread clinical application. Since 1985, PCLI assays have been performed by an efficient immunofluorescence technique utilizing bromodeoxyuridine (BrdUrd) incorporation into DNA [10,11]. Despite being technically simple, reproducible, and rapid, the BrdUrd PCLI has not gained widespread clinical use [12].

Flow cytometry has demonstrated clinical utility in the diagnosis of malignant hematologic disease, but its clinical usefulness is limited for cell kinetic measurements [13–15]. In plasma cell proliferative disorders, flow cytometry has been used for measuring DNA content (i.e., ploidy status) of monoclonal plasma cells [16–20]. A dual staining technique, DNA and cytoplasmic immunoglobulin (cIg), has been used for ploidy analysis [18,20]. A flow cytometry protocol with CD38 and DNA staining has been proposed to determine S-phase fraction of bone marrow plasma cells [21], and this technique has been shown to give prognostic information analogous to the PCLI [22].

The following is a report of 210 patients at time of study entry on Eastern Cooperative Oncology Group (ECOG) treatment protocol E9486 who had adequate DNA/cIg flow cytometry and greater than 20% bone marrow plasma cells allowing reliable measurement of DNA S-phase %. Our analysis demonstrates that the S-phase % of the light-chain-restricted cIg-positive cells has prognostic significance with respect to overall survival in patients with newly diagnosed multiple myeloma.

METHODS AND MATERIALS

Treatment Protocol

In December 1987, ECOG treatment trial E9486 began accruing patients with newly diagnosed multiple myeloma. Patient eligibility criteria and the treatment schema for this phase III trial has been published [23]. Patients were concomitantly enrolled on an ancillary laboratory study (ECOG E9487). The rationale of this study was to perform a detailed sequential analysis of peripheral blood and bone marrow in patients with multiple myeloma, to define immune parameters in relationship to clinical status, and to determine factors that would predict survival and response to therapy.

Collection of Bone Marrow Samples

A 3 ml sample of bone marrow aspirate was collected not more than 2 weeks before randomization/registration to the study. The bone marrow aspirate was immediately placed in a tube containing heparin and lyophilized bromodeoxyuridine and thoroughly mixed. The tube was then incubated at 37°C for 1 hr. 0.1 ml of 1 M thymidine (stopping solution) was then added to each tube. The tube was shipped in wet ice by overnight express to the Cell Kinetic Laboratory at the Mayo Clinic. Bone marrow mononuclear cells were collected by a Ficoll-hypaque separation. PCLI and percent bone marrow plasma cells were determined utilizing a previously published protocol [10,11]. The PCLI and S-phase analysis were done on the same bone marrow sample.

Flow Cytometry Procedure

Mononuclear cells were dual stained for kappa (κ) or lambda (λ) cIg (in separate tubes) and DNA content as follows. Mononuclear cells (1×10^6 per tube) were washed with phosphate-buffered saline (PBS), centrifuged, and supernatant removed. The cells were fixed and made permeable by adding 1 ml of 30 μg/ml lysolecithin in 1% paraformaldehyde solution to each tube, then incubated at 4°C for 30 min. The cells were centrifuged and supernatant removed. After the cells were washed with PBS, 100 μl of fluorescein isothiocyanate (FITC) conjugated anti-κ or λ antibody (1:100 dilution) was added to the appropriate tubes. Cells were incubated again at 4°C for 30 min. The cells were isolated and washed again. 100 μl of RNAase (1:10 dilution) was added to the cells and incubated for 30 minutes at 37°C and 5% CO_2 atmosphere. 1.5 mL of propidium iodide (0.25 μg/ml) solution was added to the tubes and incubated at room temperature for 0.5–2 hr before running the samples on FACS scan (Becton-Dickinson, San Jose, CA). 10,000 events were collected per run. The cIg-κ versus PI and cIg-λ versus PI dot plots were used for ploidy determination and the data were stored electronically. All patients had 10,000 ungated events collected.

Determination of S-Phase Fraction

The cIg-κ/λ versus PI dot plots were analyzed and S-phase fraction of the cIg positive monoclonal plasma cells was determined using WIN List and ModFit software programs (Verity Software House, Topsham, ME). The gate for the cIg-positive cells was selected by first reviewing the cIg-κ or λ versus PI dot plot that did not contain the light-chain-restricted population of plasma cells. This negative population was used to define the threshold for fluorescein positivity. Now the cIg-κ or λ versus PI dot plot that contained the light-chain-restricted population of cIg-positive cells was reviewed, and the S-phase fraction percentage was collected for the cells contained within the previously drawn gate. The ModFit program utilized a rectangular mathematical model for calculating the S-phase fraction. This method gave an objective measurement of the S-phase % of the cIg-positive light-chain-restricted plasma cells.

In 1993, a consensus panel published guidelines for the clinical use of flow cytometry in DNA analysis [24].

TABLE I.

Characteristic	E9486 patients with S-phase data *n* (%)	All eligible 9486 patients *n* (%)
Age		
<65	117	353
≥65	93 (44%)	275 (44%)
Hemoglobin (gm/dl)		
<10	91	230
≥10	119 (57%)	398 (63%)
Creatinine (mg/dl)		
<2	173	510
2–5	37 (18%)	118 (19%)
β_2-microglobulin (mg/ml)		
<2.7	54	159
≥2.7	154 (74%)	374 (70%)
Protein type		
IgG	119 (60%)	352 (58%)
IgA	52 (26%)	156 (26%)
Light-chain only	28 (14%)	93 (15%)
C-reactive protein (mg/dl)		
<2	180	455
≥2	26 (13%)	75 (14%)
Calcium (mg/dl)		
<12.0	196	579
≥12.0	14 (7%)	40 (7%)
Albumin (mg/dl)		
<3.0	52	134
≥3.0	157 (75%)	475 (78%)
PCLI (%)		
<1.0	140	354
≥1.0	64 (32%)	146 (29%)
S-phase (%)		
<2.0	38	
≥2.0	172 (82%)	

Because the coefficient of variation (CV) of the G_0 peak affects the accuracy of S-phase determination, it was suggested that the CV of the tumor G_0 population be <8%. The panel suggested that DNA histograms with >20% background, aggregates, or debris (BAD) were unsatisfactory for S-phase measurement.

Patients

628 eligible patients were enrolled in E9486. 500 patients had bone marrow samples successfully analyzed for PCLI. 349 patients with PCLI data had flow cytometry data, and 321 of these had DNA histograms suitable for examination according to published flow cytometry guidelines.

Only patients with ≥20% bone marrow plasma cells were considered adequate for S-phase determination because identifying the cIg-positive population and analytically separating them from the normal hematopoietic cells was difficult and subjective in patients with lower percentages of plasma cells. Thus, 210 patients with ≥20% bone marrow plasma cells, treated on protocol with complete follow-up were determined to have adequate S-phase data.

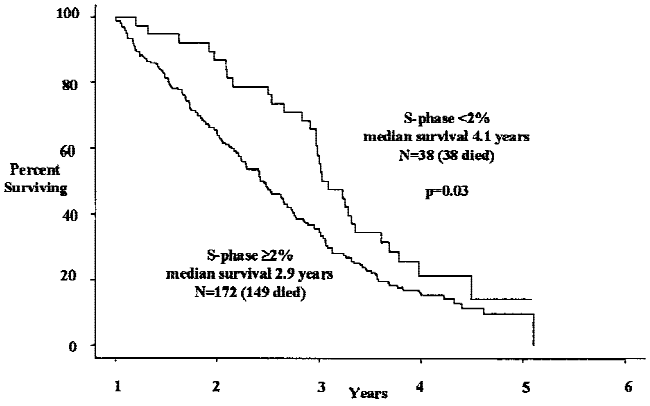

Fig. 1. Kaplan-Meier curves for 210 eligible patients with measurement of S-phase % who had satisfactory flow histograms and >20% plasma cells. Survival is plotted for those with high versus low S-phase %.

Table 1 contains clinical characteristics of this group compared to the 628 patients eligible for E9486. In some cases, the number of patients does not add up to the total eligible patients due to missing laboratory data. We concluded that the characteristics of the 210 patients with S-phase data were similar to the whole 628 patient group eligible for clinical trial E9486.

Statistical Methods

Survival was computed from the time of study registration to the date of death or data was censored at the date last known alive. Survival curves were calculated using the method of Kaplan-Meier [25] and compared by the log rank test. Spearman correlation between S-phase and PCLI was used. The proportional hazards model was used to adjust for one or more explanatory variable simultaneously when assessing survival [26]. All *P* values were two-sided.

In order to dichotomize S-phase, classification trees were performed in order to select a cutoff point for S-phase. With this method, survival times through martingale residuals were repeatedly divided into subgroups at levels that identify the biggest differences in survival [27]. There was no cross-validation on a test sample because of the size constraints of our sample size.

RESULTS

Survival Data

S-phase was modeled as a dichotomous variable for easier clinical interpretation. For the 210 patients with adequate S-phase data, median overall survival for those with S-phase <2% versus ≥2% values was 4.1 vs. 2.9 years ($P = 0.032$) (Fig. 1).

Relationship of S-Phase and PCLI

For these 204 patients with both measurements, the S-phase % had a statistically significant correlation with

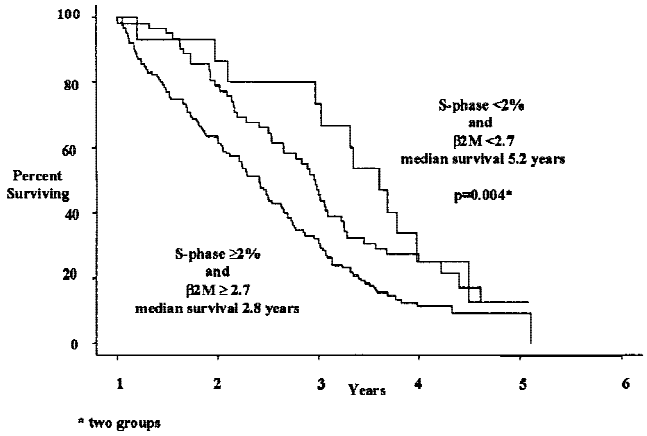

Fig. 2. Kaplan-Meier curves for the 202 eligible patients with data for both S-phase and serum β_2-microglobulin (β2M). Survival is plotted for three groups according to their β2M and S-phase %. The lower curve includes patients with high values for both; the upper curve includes patients with low values for both; the middle curve represents patients with mixed results.

PCLI. The Spearman correlation coefficient (ρ) was 0.32 (95% confidence intervals of 0.18, 0.43, $P < 0.001$).

PCLI and S-phase fraction were both prognostic for survival. Median survival for the 500 patients on E9486 with low (<1%) versus high (≥1%) PCLI was 4.0 vs. 2.4 years ($P < 0.001$). More patients had increased marrow plasma cell proliferative activity using the S-phase % than the PCLI (82% vs. 29%). In a multivariate analysis model including patients with both PCLI and S-phase fraction, only PCLI was independently prognostic. S-phase % had independent prognostic significance only if all 321 patients with flow cytometric S-phase % data were included and if PCLI was excluded from the multivariate model.

Serum β_2-microglobulin is an established prognostic factor for myeloma [28–31]. A combination of β_2-microglobulin and S-phase fraction was able to distinguish patients into three separate prognostic groups. Two patients did not have β_2-microglobulin evaluated. Median survival for all 208 patients with low (<2%) S-phase fraction and low serum β_2-microglobulin (<2.7 mg/dL) was 5.2 years vs. 2.8 years for patients with high S-phase fraction and elevated serum β_2-microglobulin ($P = 0.004$) (Fig. 2). Patients with either low S-phase fraction and elevated serum β_2-microglobulin or high S-phase fraction and normal β_2-microglobulin had a median survival of 3.9 years.

DISCUSSION

In this report, we showed that both microscope based PCLI and flow cytometric S-phase % are prognostic for survival in patients with multiple myeloma. Both the PCLI and S-phase % specifically measure the proliferative activity of the light-chain-restricted cIg-positive population. One advantage of the cIg/DNA dual-staining flow cytometry technique over the PCLI for S-phase determination is its rapidity and absence of a requirement for pre-incubation of the sample with BrdU. However, we found the determination of the S-phase fraction is imprecise when the relative proportion of monoclonal plasma cells in the bone marrow aspirate is low. This is because high S-phase normal hematopoietic cells contaminate and cause false elevation of the S-phase % measurement of the slowly proliferating malignant plasma cells. Measuring the S-phase fraction of solid tumors, breast, prostate, and colon cancer is less of a problem because the S-phase fraction in the contaminating normal cells is relatively low [22,32].

The consensus conference on clinical DNA cytometry content in malignancy suggested as a guideline that 15–20% of cells in a specimen must contain the neoplastic cells in question [24]. Selecting patients with ≥20% plasma cells for analysis of the S-phase % in this study conforms to these guidelines. Furthermore, the lower the percentage of marrow plasma cells, the higher the likelihood of including slowly proliferating normal, polyclonal, plasma cells within cIg-positive population. In our study, when estimating the percent of polyclonal plasma cells in patients with ≥20% marrow plasma cells, no patient had >4% contamination of polyclonal plasma cells in the cIg population. Seven patients had between 2–4% contamination. The rest of the patients had <2% contamination, the vast majority (>80%) of patients had ≤1% contamination. The estimation of the percent polyclonal plasma cell contamination is based on the κ/λ ratio obtained during the PCLI measurement, and assumes that the ratio of the polyclonal plasma cells to the monoclonal plasma cells for a given light chain isotype is equivalent to the κ/λ ratio. A technique that might be of benefit is to run the patient sample until at least 1,000 cIg positive events are collected with a live gate. Even with this method, the risk of contaminating normal plasma cells in the measurement increases especially as the bone marrow plasma cell content approaches normal.

Determining the S-phase % of marrow plasma cells in bone marrow with lower percentage of plasma cells is a problem not isolated to the DNA/cIg flow cytometry technique. In the myeloma staging system proposed by San Miguel et al. [22] using DNA/CD38 staining, patients with lower percentages of plasma cells had extra CD38 positive events collected. Despite this, 11 of the 131 (8.4%) cases they analyzed did not have appropriate data for S-phase analysis. These patients were not included in the survival analysis for their proposed staging system. Likewise, in the present study, 28 of the 349 (8.0%) patients did not have adequate data for S-phase

analysis but another 111 patients had less than 20% plasma cells.

The cutoff for low versus high proliferative activity used in this study of <2% and ≥2% was chosen prospectively by regression tree analysis. The flow cytometry and staging system of San Miguel et al. [22] used a compatible cutoff of <3% and ≥3%, which was the median S-phase fraction of the CD38 positive cells. Similar to San Miguel's data, the median S-phase fraction in this study was 3.7% for all patients and 3.1% for patients with ≥20% marrow plasma cells.

One would expect that the correlation between S-phase fraction and PCLI to be better than that observed in this study, and that a similar percentage of patients should have high proliferative activity. However, disparity between S-phase fraction and labeling index has been seen in other hematologic malignancies [33,34]. Although both S-phase fraction and PCLI are both measurements of proliferative activity, these tests measure proliferation differently [35]. S-phase fraction is a measurement of how many cells are proliferating at a given moment in time, whereas PCLI is a measurement of proliferative rate; i.e., how many plasma cells are proliferating and incorporating BrdUrd into DNA during the 30 min incubation period. Cells moving relatively slowly through S-phase may not incorporate sufficient BrdUrd, explaining the lower values obtained for PLCI compared to S-phase measurement.

Based on the data in this study, we conclude that the S-phase fraction of cIg positive plasma cells gives statistically significant prognostic information for patients with newly diagnosed myeloma. However, the S-phase fraction does not add new information to a model that contains previously reported prognostic parameters such as plasmablastic morphology, soluble IL-6 receptor, and serum β_2-microglobulin unless the more established measurement of PCLI is excluded from the model and the numbers of cases increased by including 111 cases with suspected results because they had <20% plasma cells. Because flow cytometry is widely available in clinical laboratories and is less labor intensive than immunofluorescence microscopy, it is likely that the measurement of the plasma cell S-phase % may someday become a more useful procedure than PCLI for prognosis in multiple myeloma. We believe that improved flow cytometric procedures must be developed before large prospective studies of the diagnostic and prognostic value of flow cytometric estimates of S-phase %.

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